Differential Regulation of Early Response Genes and Cell Proliferation Through the Human Granulocyte Macrophage Colony-Stimulating Factor Receptor: Selective Activation of the c-fos Promoter by Genistein

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Granulocyte macrophage colony-stimulating factor (GM-CSF) binds to the high-affinity GM-CSF receptor (GMR) consisting of α and β subunits and induces rapid tyrosine phosphorylation, activation of early response genes, and proliferation of hematopoietic cells. The α subunit is the primary cytokine binding component and the β subunit is required for high-affinity binding as well as for signal transduction. Using tyrosine kinase inhibitors and cytoplasmic deletion mutants of the β subunit, we obtained evidence that there are at least two distinct pathways downstream of the GMR in BA/F3 cell, one which is essential for proliferation, leads to the c-myc gene activation, and is sensitive to herbimycin and genistein. Activation of this pathway depends on the cytoplasmic region between amino acid positions 455 and 517 of the β subunit. The second pathway, which leads to activation of c-fos and c-jun genes, is only partially sensitive to herbimycin, is resistant to genistein and depends on the region between amino acid positions 626 and 763 of the β subunit. Unexpectedly, the c-fos mRNA induction was augmented by genistein. The enhanced expression of c-fos mRNA by genistein also occurred with stimulation with cAMP, PMA, or EGF in NIH3T3 cells. It thus seems likely that genistein affects a common pathway downstream of these signals.

INTRODUCTION

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that regulates growth and differentiation of various hematopoietic cells, including early hematopoietic progenitors (recent review by Arai et al., [1990]). GM-CSF exhibits biological activities similar to those seen with interleukin (IL)-3 (Arai et al., 1990; Watanabe et al., 1991) and both act in a species-specific manner between mouse and human (Lee et al., 1985; Yang et al., 1986). IL-3, which is produced mainly by activated T cells and mast cells, appears to be important for "inducible" hematopoiesis associated with immune and inflammatory reactions, whereas GM-CSF is produced by a wide variety of cells, including macrophages, endothelial cells, and fibroblasts in addition to activated T cells and mast cells and is likely to be involved in normal as well as "inducible" hematopoiesis (Arai et al., 1990).

Molecular cloning of the cytokine receptor cDNAs demonstrated that the high-affinity GM-CSF receptor (GMR) is composed of two subunits α and β, both of which are the members of the cytokine receptor family (see Miyajima et al., 1992 for a review). The β subunit of the GMR is shared by the IL-3 receptor (IL-3R) and the IL-5 receptor, whereas α subunits are unique to each (Hayashida et al., 1990; Itoh et al., 1990; Kitamura et al., 1991; Hara and Miyajima, 1992). The β subunit is
required for the high-affinity binding of cytokines and is also essential for signal transduction. Thus, the shared receptor subunit with signaling function provides a molecular basis that would account for the overlapping biological activities between GM-CSF and IL-3 (Miyajima et al., 1992). Although GM-CSF as well as IL-3 induce rapid tyrosine phosphorylation of several cytoplasmic proteins, including the β subunit (Isfort et al., 1988a,b; Kanakura et al., 1990), neither tyrosine kinase activity nor the sequence homologous to tyrosine kinases has been found in the cytoplasmic regions of α and β subunits of GM or IL-3R (Hayashida et al., 1990; Itoh et al., 1990; Kitamura et al., 1991; Hara and Miyajima, 1992). These observations suggest that an additional molecule(s) with tyrosine kinase activity is involved in the signal transduction pathway of GM-CSF or IL-3, a notion consistent with the observation that GM-CSF or IL-3 activates several tyrosine kinases, including the src family tyrosine kinases (Torigoe et al., 1992; Hanazono et al., 1993). These results also suggest that multiple or different kinases are activated, depending on the nature of cells types or functions. However, the correlation between GM-CSF function and activation of tyrosine kinase is unclear and the roles of tyrosine kinase(s) in signal transduction via GM or IL-3 are not well understood. It is also not clear whether these tyrosine kinases interact directly with GM-CSF or indirectly through an unidentified component(s).

Protooncogenes c-fos, c-jun, and c-myc are members of the early response genes and are activated by various stimuli including IL-3, GM-CSF, and other growth factors (Greenberg and Ziff, 1984; Greenberg et al., 1985; Watanabe et al., 1993). While c-fos and c-myc genes are involved in cell growth (Holt et al., 1986; Marcu et al., 1992), the exact role of these proto-oncogenes in signal transduction of cytokine receptors remains elusive. We and other groups reported that the high-affinity human GM (hGM) reconstituted in a pro-B cell line BA/F3 cells or NIH3T3 fibroblasts by cotransfecting cDNAs for the α and β subunits transduces signals in response to human GM-CSF (hGM-CSF), i.e., activation of c-fos, c-jun, and c-myc proto-oncogenes and stimulation of DNA synthesis (Eder et al., 1993; Watanabe et al., 1993). Although pattern of the expression of the src family kinases differs from that in hematopoietic cells, phosphorylation of proteins of almost the same size at tyrosine residues occurred with hGM-CSF stimulation in NIH3T3 cells expressing reconstituted hGM (Watanabe et al., 1993). All these findings suggested that the hGM may be linked to signaling pathways present in fibroblasts and that the association of hGM with factors specific to hematopoietic cell lineage is not essential to transduce growth promoting signals.

In earlier work, we generated a series of cytoplasmic deletion mutants of the β subunit of hGM and found that the membrane proximal region is essential for proliferation and that the distal region is responsible for major tyrosine phosphorylation (Sakamaki et al., 1992). Interestingly however, hGM-CSF–mediated proliferation through the mutant hGMRβ subunit, which failed to induce apparent tyrosine phosphorylation, remained herbimycin A (herbimycin)-sensitive (Sakamaki et al., 1992). Thus, involvement of a herbimycin-sensitive tyrosine kinase in the essential signaling pathway may lead to cell proliferation. Here, we report evidence that GM-CSF induces multiple pathways for induction of early responsive genes: one pathway, which is essential for cell proliferation, involves c-myc mRNA induction and is sensitive to herbimycin and genistein and the other, required for induction of c-fos and c-jun mRNA, is insensitive to herbimycin and genistein.

MATERIALS AND METHODS

Chemicals, Media, and Cytokines

[3H]Thymidine and [γ-32P]ATP were from Amersham Japan. Fetal calf serum (FCS) was from Biocell Labsors (Carson, CA). Dulbecco’s modified Eagle’s medium (DMEM) and RPMI-1640 were from Nihon BioMedical Laboratories. Recombinant hGM-CSF produced in Escherichia coli was provided by Dr. R. Kastelein (DNAX Research Institute). Mouse IL-3 (mIL-3) produced by silkworm (Bombyx mori) was purified as described elsewhere (Miyajima et al., 1987). Genistein was from Wako Pure Chemical Industries. Herbimycin and G418 were purchased from Gibco Life Technologies (Grand Island, NY). Myelin basic protein (MBP), casein, N[γ-2′-O-dibutylryl-guanoine 3′-5′-cyclic monophosphate (db2cAMP) and 12-O-tetradecanoyl-phorbol-13-acetate (PMA) were from Sigma Chemical (St. Louis, MO).

Cell Lines and Culture Methods

A mIL-3–dependent proB cell line, BA/F3 (Palacios and Steinmetz, 1985), were maintained in RPMI-1640 medium containing 8% FCS, 1 ng/ml mIL-3, 100 U/ml penicillin, and 100 µg/ml streptomycin. A mouse NIH3T3 fibroblast line was maintained in DMEM containing 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Transfected BA/F3 cells or NIH3T3 expressing hGM α and β chains were grown in the same type of media but supplemented with 500 µg/ml G418.

Plasmids and Genes

The c-fos promoter-luciferase gene fusion plasmid was constructed as described previously (Watanabe et al., 1993). Rat c-jun and mouse c-fos cDNA plasmids were kind gifts from Dr. T. Curran (Roche Institute of Molecular Biology). Mouse c-myc was provided from Dr. N. Arai (DNAX Research Institute).

Transient Transfection of DNA

Individual plasmid DNA or a combination of multiple plasmid DNAs (total 5 µg) was transfected into semiconfluent BA/F3 cells by the diethyl-amino ethyl (DEAE)-dextran method and mouse NIH3T3 cells by electroporation, respectively, as described elsewhere (Watanabe et al., 1993).

Northern Blot Analysis

Northern blot analysis was performed with total cellular RNA prepared using the guanidinium thiocyanate extraction method (Maniatis et al., 1982).
Nuclear (10 threitol (PBS) tris(hydroxymethyl)aminomethane NaCl, for stimulated, as same Vol. 4, 15 min. Luciferase Assay for 15 min. One hundred SDS, sodium mIL-3 activity or MAPK Gaithersburg, 22 BA/F3 mIL-3, 24-h 2.2% NIH3T3 cells, according to the method described above. Oligonucleotide of the AP-1 within the c-jun promoter (CCGTGATGATCCCGGAA) was chemically synthesized and purified by Sephadex G-50 nick columns (Pharmacia, Upsala, Sweden) after labeling with [32P]dCTP. The extracts containing 5 μg protein were incubated with a labeled probe for 15 min at room temperature in a total volume of 15 μl containing 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, 1 mM EDTA, pH 8.0, 12.5% glycerol, 0.1% Triton X-100, 5 μg of bovine serum albumin, and 2.5 μg of poly dIdC as a nonspecific competitor. For competition experiments, non-labeled competitor DNA was incubated on ice with the extract for 15 min, before addition of the probe. The DNA-protein complexes were electrophoresed with a 4% polyacrylamide gel in 6.7 mM Tris, pH 7.5, 3.3 mM sodium acetate, and 1 mM EDTA, pH 8.0, and then gel was transferred to Whatman 3MM (Minneapolis, MN) paper, dried, and analyzed by Fuji image analyzer (model BAS-2000).

Electrophoretic Mobility Shift Assay (Gel Retardation Analysis)

Gel retardation analyses were performed with nuclear extracts prepared from BA/F3 cells, according to the method described above. Oligonucleotide of the AP-1 promoter was radiolabeled (CCGTGATGATCCCGGAA) and purified by Sephadex G-200 columns (Pharmacia, Uppsala, Sweden) after labeling with [32P]dCTP. The extracts containing 5 μg protein were incubated with a labeled probe for 15 min at room temperature in a total volume of 15 μl containing 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, 1 mM EDTA, pH 8.0, 12.5% glycerol, 0.1% Triton X-100, 5 μg of bovine serum albumin, and 2.5 μg of poly dIdC as a nonspecific competitor. For competition experiments, non-labeled competitor DNA was incubated on ice with the extract for 15 min, before addition of the probe. The DNA-protein complexes were electrophoresed with a 4% polyacrylamide gel in 6.7 mM Tris, pH 7.5, 3.3 mM sodium acetate, and 1 mM EDTA, pH 8.0, and then gel was transferred to Whatman 3MM (Minneapolis, MN) paper, dried, and analyzed by Fuji image analyzer (model BAS-2000).

RESULTS

Effects of Protein Tyrosine Kinase Inhibitors on Induction of c-fos and c-myc mRNAs

To examine the possible contribution of tyrosine kinases to GM-CSF-mediated signal transduction, we made use of the protein tyrosine kinase inhibitors, herbimycin and genistein. Herbimycin inhibits src-related kinases (Uehara et al., 1989) and genistein inhibits tyrosine kinases including the EGF receptor, pp60-v-src and pp110γag-fes (Badwey et al., 1991). We first analyzed the effects of herbimycin or genistein on GM-CSF-mediated c-fos, c-jun, and c-myc induction using Northern blot analysis of BA/F3 cells expressing hGMα and β chain (BAF/αβ) cells. BAF/αβ cells depleted of factor were stimulated with either mIL-3 or hGM-CSF in the presence of these inhibitors. Expression of the endogenous c-fos mRNA induced by either mIL-3 or hGM-CSF was only slightly inhibited by herbimycin, whereas the endogenous c-myc transcript was almost completely abolished (Figure 1). Genistein, similar to herbimycin, completely suppressed the induction of c-myc mRNA. However, unlike herbimycin, genistein markedly augmented the level of c-fos mRNA expression.

Effects of Protein Tyrosine Kinase Inhibitors on Activation of the c-fos Promoter

We reported earlier the transient transfection assay using c-fos promoter-luciferase plasmid to examine GM-CSF-
mediated c-fos mRNA activation quickly and easily (Watanabe et al., 1993). We next did experiments to observe the effects of herbimycin and genistein on GM-CSF–mediated c-fos induction, using the c-fos promoter–luciferase fusion gene in BAF/αβ cells. The c-fos promoter–luciferase plasmid that contains only the 0.4 kb-upstream region of the translational initiation site and lacks sequences that are known to destabilize c-fos mRNA, was transfected into BAF/αβ cells and the luciferase activity was measured (Figure 2). In BAF/αβ cells, herbimycin suppressed hGM-CSF–dependent or mIL-3–dependent induction of c-fos promoter luciferase activity as much as 20–30%. Unlike herbimycin, genistein augmented c-fos promoter activity induced by hGM-CSF or mIL-3 in BAF/αβ cells. These results demonstrated that the transfected c-fos promoter responds to herbimycin or genistein in a manner similar to events seen with the endogenous c-fos mRNA. They also suggest that genistein affects c-fos mRNA at the transcriptional level rather than at the post-transcriptional level. Similar analysis was made of NIH3T3 cells expressing the normal hGMR α and β subunits (NIH/αβ). In NIH/αβ cells, the c-fos promoter luciferase activity induced by FCS or GM-CSF appeared to be more sensitive to herbimycin and the luciferase activity was suppressed by as much as 60%.

Effects of Protein Tyrosine Kinase Inhibitors on Cell Proliferation

The effects of tyrosine kinase inhibitors on hGM-CSF–dependent proliferation of BAF/αβ were also examined after the incorporation of [3H]thymidine (Figure 3). Unlike the induction of c-fos mRNA, both herbimycin and genistein suppressed DNA synthesis induced by hGM-CSF or mIL-3 in BAF/αβ cells, in a dose-dependent manner. Herbimycin and genistein, at concentrations 0.5 and 10 μg/ml, respectively, completely suppressed [3H]thymidine incorporation (Figure 3). Essentially the
same results were obtained with NIH/αβ cells stimulated with either FCS or hGM-CSF. These results suggest that increase in the c-myc mRNA level, as well as the stimulation of DNA synthesis in BA/Fαβ and NIH/αβ cells via GMR, involves herbimycin- and/or genistein-sensitive tyrosine kinase(s). On the other hand, the induction of c-fos and c-jun mRNAs depends only partially on herbimycin- and/or genistein-sensitive tyrosine kinase.

Cytoplasmic Domain of the hGMRβ Chain Required for Activation of c-fos, c-jun, and c-myc Genes and DNA Synthesis

Using a series of the cytoplasmic deletion mutants of the β subunit and MTT assay, we have found that the cytoplasmic region between amino acid position 455 and 517 is essential for GM-CSF–stimulated cell growth and the distal region is responsible for the major tyrosine phosphorylation (Sakamaki et al., 1992). To define the region of the hGMRβ chain controlling DNA synthesis activity, we examined [3H]thymidine incorporation using BA/F3 cells expressing various β chain deletion mutants and the wild-type α chain of hGMR (BA/F825-455) (Sakamaki et al., 1992). As shown in Figure 4, BA/F455 was fully functional, whereas BA/F517 showed partial (16% of BA/F544) activity and BA/F455 is completely inactive. These findings suggest that the region between positions 455 and 517 is essential for DNA synthesis and the region between positions 517 and 544 is not essential but necessary for full activation. We further analyzed regions of the β chain required for activation of early response genes by Northern blot analysis with BA/F3 cells expressing various β chain mutant (Figure 5). BA/F3 cells expressing hGMRα/β induced c-fos, c-jun, and c-myc mRNA in response to either mIL-3 or hGM-CSF (Watanabe et al., 1993). Cells stably transfected with the normal hGMR α subunit and a series of the β deletion mutants were factor-depleted and stimulated with either mIL-3 or hGM-CSF. As shown in Figure 5, c-myc mRNA induction was fully functional in BA/F825 and BA/F763 and decreased ~40% in BA/F626 or BA/F544 and further decreased in stepwise manner after deletion of the β chain; the levels of c-myc mRNA in BA/F544, BA/F517, and BA/F455 were 100, 21, and 1, respectively. These results indicate that the region required for induction of c-myc mRNA correspond to the region essential for inducing proliferation signals. In contrast, the induction of c-fos and c-jun mRNAs was detected only in BA/F3 transfectants that expressed the normal β subunit; βα23 and βα23 mutants, but not βα25 or shorter β mutants. These observations provide evidence for two distinct cytoplasmic regions of the β subunit that are responsible for different signaling pathways to proto-oncogene induction.

The Region Essential for Activation of c-fos/c-jun Genes Is also Required for Formation of the AP-1 Complex

c-fos protein forms a heterodimeric complex with c-jun protein (Angel and Karin, 1991) and this complex, AP-1 binds to the AP-1 binding sequence (TGACTCA). Likewise, other proteins that belong to c-fos or c-jun protein superfamily also form a complex that binds to the AP-1 binding sequence. To examine whether or not the functional AP-1 protein would be produced in re-
response to hGM-CSF stimulation, we performed gel retardation analysis using oligonucleotide corresponding to the AP-1 sequence of the c-jun promoter. Unstimulated BAF/αβ cells showed no detectable level of AP-1 bands. The amount of AP-1 complex was not significantly changed after 30 min, but stimulation of BAF/αβ cells with 10 ng/ml of mIL-3 resulted in the marked increase of AP-1 complex after 2 h. Formation of this complex was inhibited by the addition of a 30-fold molar excess of the unlabeled wild-type AP-1 oligonucleotide. Stimulation of BAF/αβ cells with hGM-CSF (10 ng/ml) induced an AP-1 complex with mobility and kinetics similar to those induced by mIL-3 (Figure 6). The addition of mIL-3 induced the complex in all BA/F3 cells expressing hGMRβ chain mutants. In contrast, hGM-CSF failed to induce the AP-1 complex through the β626, β544, β317, and β455 mutants, thereby indicating that the region between positions 763 and 626 is essential to induce formation of the AP-1 complex. As expected, this region corresponds to that required for induction of c-fos and c-jun mRNAs.

**Effect of Genistein on the Kinetics of Induction of c-fos, c-jun, and c-myc mRNAs**

We next used Northern blots to examine the effects of genistein on the kinetics of appearance and/or disappearance of c-fos mRNA (Figure 7). In the absence of an inhibitor, c-fos mRNA appeared 15 min after exposure to hGM-CSF and disappeared after 60 min. Marked augmentation of c-fos mRNA level by genistein was observed at 15 min. Sixty minutes after stimulation, the c-fos mRNA level sharply declined, even in the presence of genistein. In contrast, c-myc mRNA began to accumulate 15 min after GM-CSF treatment and reached a maximum level at 60 min, a plateau level was sustained for ≥2 h after stimulation. Genistein suppressed the c-myc mRNA level and this suppression continued for ≥2 hr after stimulation. These results are interpreted to mean that genistein exerts effects without affecting kinetic parameters for c-fos mRNA.

**Effects of Genistein and Herbimycin on c-fos Promoter Activation in Response to Various Stimuli**

To examine whether or not genistein would affect the c-fos mRNA level in an hGM-CSF-specific or cell-spe-
sible involvement of several kinases with activities that may control expression of the c-fos gene. MAPK is known to be activated by various growth factors including mIL-3 (Welham et al., 1992). CKII positively regulates transcription of the c-fos gene through phosphorylation of serum response factor (SRF) (Manak et al., 1990; Marais et al., 1992). Total protein extracts of NIH/αβ or BAF/αβ cells stimulated for 5 min in the presence or absence of genistein were separated by SDS-polyacrylamide gel containing MBP or casein as a substrate for MAPK and CKII, respectively. Analysis of each kinase activity was made by an in-gel-phosphorylation assay (Gotoh et al., 1990) as described in MATERIALS AND METHODS. As shown in Figure 9, in NIH/αβ, MAPK activity, which was undetectable before stimulation, was markedly activated by stimulation with either EGF, FCS or hGM-CSF, and slightly by PMA. Genistein did not affect the levels of either basal or activated MAPK activities. In contrast, CKII activity was detectable without stimulation and was not appreciably affected by various stimuli such as FCS, hGM-CSF, EGF, or PMA. Here too, genistein did not affect CKII activity. Essentially the same results were obtained with BAF/αβ cells stimulated with either hGM-CSF or mIL-3.

**DISCUSSION**

**Distinct Pathways for Induction of c-fos/c-jun and c-myc mRNAs**

The results obtained using tyrosine kinase inhibitors indicate that hGM-CSF activates two distinct signaling pathways that lead to the activation of nuclear proto-oncogenes, one for induction of c-fos/c-jun mRNAs and the other for induction of c-myc mRNA. In BAF/αβ cells, the induction of c-myc mRNA is completely suppressed by tyrosine kinase inhibitors, herbimycin and genistein, whereas c-fos/c-jun mRNAs are only partially suppressed or even augmented by the same tyrosine kinase inhibitors. Interestingly, GM-CSF-induced cell proliferation was also sensitive to either herbimycin or genistein, in a manner similar to that seen with the induction of c-myc mRNA. In NIH3T3 fibroblasts, herbimycin inhibited EGF-induced c-fos-luciferase activity, whereas it only partially suppressed the levels of c-fos and c-jun mRNAs induced by hGM-CSF, indicating that the lack of sensitivity of c-fos mRNA induction to herbimycin is unique to GM-CSF and is not restricted to hematopoietic cells. Deletion mapping of the β subunit revealed that the region required for activation of c-fos and c-jun genes is separable from the region essential for induction of c-myc mRNA and corresponds to the region required for the formation of the functional AP-1 complex. Thus, complete suppression by tyrosine kinase inhibitors of both cell proliferation and of c-myc mRNA accumulation induced by hGM-CSF is consistent with the deletion analysis of the β subunit. However, hGMR, which consists of wild type α chain and mutant β chain carrying deletion at amino acid positions 555-517, did not induce c-fos-luciferase activity in both BA/F3 and NIH3T3 cells, suggesting that the region covering positions 626-763 is essential but is not sufficient to induce c-fos mRNA. They also suggest that domains for c-fos and c-myc gene activation are not completely independent. However, we could not exclude the possibility that this internal deletion disrupted the overall structure of the β chain. Studies are ongoing to elucidate the requirement of membrane proximal region using a series of mutants having point mutation within this region.

GM-CSF induces c-myc mRNA and cell proliferation through the β317, β546, and β826 mutant receptors, even though it failed to induce c-fos mRNA and AP-1 complex formation under the condition of cultures we used. These findings differ from the report of a close correlation between the induction of the c-fos mRNA and proliferation (Holt et al., 1986). As the standard culture medium for BA/F3 cells contains 10% FCS, it is possible that components in the serum induce signals that complement the requirement for c-fos mRNA induction.

Our findings that c-fos and c-myc mRNAs are induced through different regions of the GMR/β chain are similar to the results described for the IL-2Rβ chain (Hatakeyama et al., 1989, 1992). However, in the IL-2R system, tyrosine kinase, which interacts with the acidic region of the IL-2Rβ chain, may be required to induce c-fos and c-jun mRNAs (Minami et al., 1993). In the hGMR system, it may be that tyrosine kinase is not essential for activation of these genes. Alternatively, tyrosine kinase(s), which is herbimycin- or genestein-insensitive may be involved in the activation of c-fos/c-jun genes. It has been suggested that Ras protein–stimulated c-fos gene expression at the level of transcription (Schoenthal et al., 1988). In BA/F3 cells, mIL-3 induces a rapid conversion of Ras protein from GDP-bound form to GTP-bound form, a process that is inhibited by herbimycin (Satoh et al., 1992). Taken together, these results would suggest that herbimycin-sensitive tyrosine kinase is involved in the pathway to regulate Ras protein and thereby to activate c-fos gene. It remains to be deter-
minded whether or not the same tyrosine kinase regulates both c-myc mRNA and Ras protein. However, our results that c-fos mRNA induction by hGM-CSF is herbinycin-resistant suggest other possibilities for the roles of Ras protein in c-fos mRNA induction. These include 1) activation of Ras protein from GDP-bound form to GTP-bound form is not essential c-fos mRNA induction, 2) multiple pathways including Ras-dependent and -independent pathways exist for the induction of c-fos mRNA, or 3) Ras protein is essential but residual Ras protein complexed with GTP is sufficient for c-fos mRNA induction.

Genistein Upregulates Transcription of c-fos Gene Induced by Multiple Stimuli: Possible Mechanism

Another interesting finding in this work is that genistein markedly augmented the c-fos mRNA level induced by hGM-CSF, in both BAF/αβ and NIH/αβ cells and without affecting the levels of c-jun and c-myc mRNAs. We attempted to define the unique region within the hGMRβ chain that responds to genistein resulting in the augmented response to hGM-CSF. Genistein augmented the response of c-fos promoter to hGM-CSF, even in BA/FGM cells expressing a minimum region of β chain to activate c-fos gene. These results suggest the possibility that the region of hGMRβ chain responding to genistein is inseparable from that required for c-fos mRNA induction. Furthermore, the effects of genistein do not seem to be restricted to GM-CSF. As genistein augmented the level of c-fos mRNA induced by hGM-CSF, EGF, protein kinase A (PKA) or protein kinase C (PKC), the target(s) of genistein appears to involve a component(s) of the signal-transducing pathways common to these signals. Alternatively, genistein may affect the c-fos promoter activity independent of these signaling pathways.

The level of c-fos mRNA is regulated by transcriptional and post-transcriptional mechanisms. The c-fos mRNA is unstable and is rapidly destroyed by at least two distinct mRNA degradation pathways: One recognizes a 75-nucleotide AU-rich instability determinant (ARE) located within the 3′ untranslated region (UTR) (Shyu et al., 1989), and the other recognizes a destabilizing element(s) present in the protein-coding region (Chen et al., 1992). Further analyses using a c-fos-luciferase plasmid showed that the response to genistein is mediated through the 5′-flanking region of c-fos promoter, thereby indicating that these degradation mechanisms of c-fos mRNA are not involved.

The c-fos protein itself can repress the level of c-fos mRNA and serum responsive element (SRE) site of the c-fos promoter appears to be involved in this phenomenon (Lucibello et al., 1989). This transrepression is regulated by altering the phosphorylation state of the carboxy terminal region of the c-fos protein (Ofir et al., 1990). It is tempting to speculate that genistein, by inhibiting the phosphorylation of c-fos protein, eliminates this repression. Kinetic analysis revealed that the rates of induction and disappearance of c-fos mRNA is unaffected by genistein. Mouse EC cell line P19 produces very low levels of endogenous c-fos protein. Genistein also augmented the c-fos-luciferase activity in this line expressing hGMRα and -β chains in response to hGM-CSF. In addition, gel retardation assays using SRE oligonucleotides showed no detectable change of SRE complex formation in response to either GM-CSF or IL-3 regardless of the presence or absence of genistein. These results suggest that genistein does not appreciably affect the suppression of c-fos mRNA induction and that the action of genistein does not appear to involve the release from autosuppression by c-fos protein.

To search for the possible target(s) of genistein, we examined the possibility that genistein, which has broad targets (Huang et al., 1992), may affect kinase(s) other than tyrosine kinase(s). The close relationship between MAPK and c-fos mRNA induction has been deduced from observations that MAPK stimulates phosphorylation of a positive transcription factor for the c-fos promoter (Gille et al., 1992). However, genistein did not significantly affect MAPK activity in response to various stimuli, including GM-CSF, thereby suggesting that genistein exerts its action either downstream or independent of MAPK. A positive regulation of the c-fos promoter activity by CKII has been suggested (Manak et al., 1990; Marais et al., 1992), but our results suggested that CKII was not the target of genistein. While more work is necessary to elucidate the mechanism by which genistein enhances the level of c-fos mRNA, the findings described in this paper that distinct regions of GMRβ chain are involved in the regulation of c-fos and c-myc mRNAs which show different response to tyrosine kinase inhibitors will pave the way toward elucidation of signal transduction events downstream of GMR.

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Signal Transduction of the Human GM-CSF Receptor


